THE STIMULATION OF GLOBIN SYNTHESIS BY HEME*

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Heme has been shown to stimulate the synthesis of globin in reticulocytes of iron-deficient rabbits[1, 2] and in the nuclei of avian erythrocytes.[3] We wish to report studies on the mechanism by which heme may regulate the synthesis of globin. When reticulocytes of iron-deficient rabbits are incubated with valine-C^{14} in the presence of added hemin, there is an increase in the size and proportion of polyribosomes, in the specific activity of the polypeptide chains attached to them, and in the specific activity of the soluble hemoglobin.

Methods.—New Zealand rabbits were maintained on a diet low in iron (Nutritional Biochemical Corp.) and glass-redistilled water and were bled repeatedly to maintain a hematocrit below 20%, a reticulocyte count of 10–15%, and a serum iron concentration of less than 40 μg per 100 ml of serum.

Studies on the synthesis of soluble hemoglobin were performed as described previously.[1] When polyribosomes were isolated, the incubation mixture contained 1.0 ml of the packed cells and 1.0 ml of medium. Freshly prepared hemin[4] was added to a final concentration of 8.5×10^{-5} *M*. An equal volume of buffer solution was added to the control flasks. The flasks were flushed with 95% O_2–5% CO_2 for 5 min, stoppered, and incubated at 37°C without shaking. At the end of 30 min, 20 μc of L-valine-U-C^{14}, of specific activity 185 mc/mM (New England Nuclear Corp.), were added and the cells incubated for an additional 2 min. The reaction was stopped and the cells were lysed by pouring the incubation mixture into 4 vol of ice-cold 0.005 *M* $MgCl_2$ with 0.006 *M* mercaptoethanol, pH 7.4. One volume of 1.5 *M* sucrose 0.15 *M* KCl and 0.006 *M* mercaptoethanol, pH 7.4, was added slowly, with stirring. The stroma was removed by centrifugation at 27,000 $\times$ *g* for 10 min. The clear supernatant was centrifuged at 105,000 $\times$ *g* for 2 hr in a Spinco model L ultracentrifuge to obtain a ribosomal pellet. The pellet was rinsed 3 times with cold RSB (0.01 *M* tris, pH 7.4, 0.01 *M* KCl, 0.0015 *M* $MgCl_2$) described by Warner, Knopf, and Rich,[5] and then homogenized in 1.5 ml of RSB by several hand strokes of an homogenizer fitted with a loose Teflon pestle. The ribosomal suspension was centrifuged at 27,000 $\times$ *g* for 10 min and the absorbancy at 260 mμ determined. From 0.7 to 1.0 ml of material, adjusted to contain an equal amount of ribosomal protein from control cells and from cells incubated with hemin, was layered on 28 ml of a linear sucrose density gradient (7.5–30% sucrose w/v in RSB). After centrifugation at 25,000 rpm in the SW25 rotor (Spinco Div., Beckman Instrument Co.) for 2.5 hr, the gradients were analyzed for absorbancy at 260 mμ in a Gilford recording spectrophotometer (Gilford Instrument Laboratories, Inc.), and 1.0 ml fractions were collected for measuring radioactivity. To each 1.0 ml fraction, 0.1 mg of bovine serum albumin, 5 mg of L-valine, and 1.0 ml of 10% trichloroacetic acid were added. The precipitates were collected on Millipore filters, washed three times with 5% trichloroacetic acid, and counted in a Nuclear-Chicago gas-flow counter. When the reticulocyte count exceeded 25%, it was occasionally possible to eliminate the concentration of ribosomes in a pellet. In these experiments the cells were lysed in 8 vol of RSB, and 1.0 ml of the stroma-free lysate was layered directly on sucrose gradients. A third method[6, 7] of examining polyribosomes was used in experiments in which six gradients were prepared simultaneously. In these experiments the cells were lysed in 4 vol of RSB, and the stroma was removed by centrifugation. The lysate was then brought to pH 5 with 1 *N* acetic acid, the resulting precipitate was collected by centrifugation at 4000 $\times$ *g* for 5 min and redissolved in RSB containing 0.05 *M* tris buffer (pH 7.8). All operations were performed at 4°C. This procedure concentrates the ribosomes and preserves the structure of polyribosomes while eliminating much of the soluble hemoglobin, which does not precipitate at pH 5.

Measurement of the polyribosome portions of the sucrose gradient patterns and of the specific activity of the polypeptide chains associated with them was performed by planimetry of the curves of OD 260 and of radioactivity.

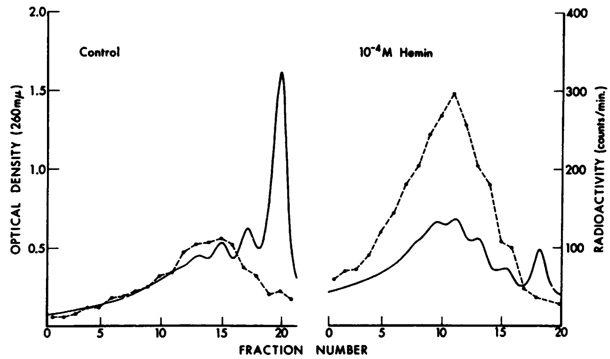

FIG. 1.—Incubation without added hemin (control) and with added hemin for 30 min. ———, Optical density; - - - -, radioactivity.

Results.—The figures present analyses of sucrose gradients of ribosomes from iron-deficient reticulocytes incubated under various conditions, followed by pulse labeling with valine-C^{14} for an additional 2 min. The effects of hemin (1×10^{-4} *M*) on the pattern of ribosomal distribution and function in reticulocytes are shown in Figure 1. There is a shift in distribution so that there are fewer single ribosomes and more of the heavier polyribosomes. There is also more radioactivity in the polypeptide chains attached to the polyribosomes when the cells are incubated in the presence of hemin. Similar results are obtained when ferrous ammonium sulfate (1×10^{-4} *M*) is used instead of hemin. In Table 1 the specific activity of the polypeptide chains attached to the polyribosomes, calculated as the ratio of total radioactivity to total polyribosomal optical density, is compared with the measured specific activity of soluble hemoglobin obtained after incubating an aliquot of the same cells for 4 hr at 37°C in the presence and absence of added hemin. Hemin causes a marked increase in the specific activity of the soluble hemoglobin and of the nascent chains on the polyribosomes. The increase in the proportion of polyribosomes which occurs on incubation with hemin is shown in Table 2.

Both hemin and iron stimulate globin synthesis in the iron-deficient reticulocyte.[8, 9, 1] To differentiate these effects, experiments were performed with

TABLE 1

EFFECTS OF ADDED HEMIN ON THE SYNTHESIS OF GLOBIN

Expt. no.	Radioactivity in Soluble Hemoglobin (cpm/mg) Control	Hemin added	Per cent increase	Radioactivity in Polypeptide Chains Attached to Polyribosomes (Total cpm/total polyribosomal OD) Control	Hemin added	Per cent increase
1	890	1833	106	0.97	1.82	87
2	966	3070	218	1.47	2.84	93
3	—	—	—	1.36	2.34	72

TABLE 2

EFFECT OF ADDED HEMIN ON POLYRIBOSOME FORMATION

Expt. no.	Total Ribosomal OD (cm^2) Control	Hemin	Percentage OD as Polyribosomes Control	Hemin
1	97.4	99.0	65	88
2	111.7	79.3	49	70
3	81.3	76.1	61	82

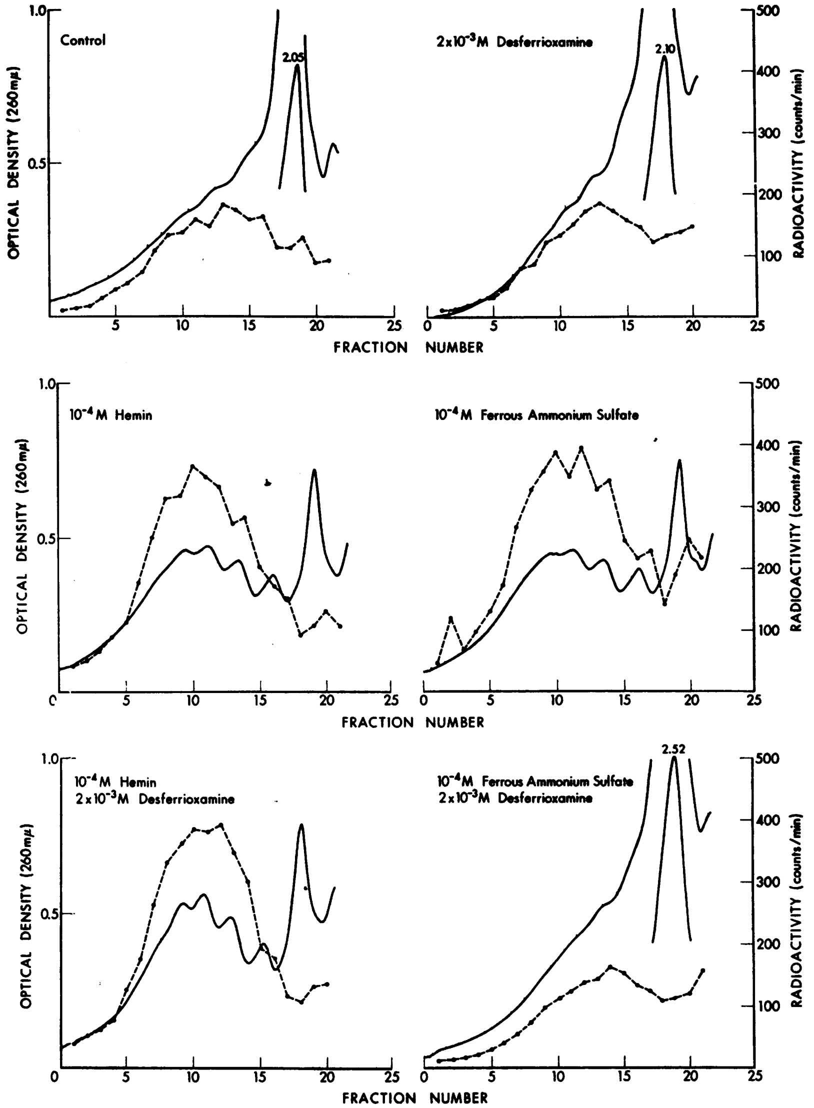

FIG. 2.—Incubation for 30 min with no additions (control) or with the compounds as indicated. ————, Optical density; - - - -, radioactivity.

the iron chelating agent, desferrioxamine methane sulfonate (Desferal obtained from Dr. W. B. Westlin, Ciba Pharmaceutical Co.). This compound, at a concentration of 2×10^{-3} *M*, inhibits globin synthesis in these cells and causes a breakdown of polyribosomes to single ribosomes. As shown in Figure 2, these effects can be prevented by the simultaneous addition of 1×10^{-4} *M* hemin but not of 1×10^{-4} *M* ferrous ammonium sulfate. The results demonstrate that hemin acts directly to stimulate globin synthesis on polyribosomes and does not serve merely as a source of iron.

Cobalt has been shown to stimulate the synthesis of globin,[8, 10] while inhibiting the synthesis of heme.[10] Figure 3 reveals that as with hemin or iron, the addition of

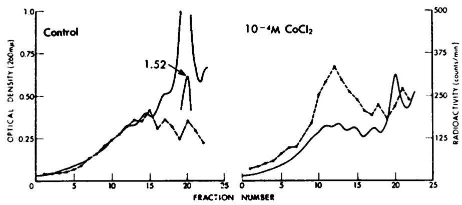

FIG. 3.—Incubation for 30 min with no additions (control) and with added cobalt. ————, Optical density; – – – –, radioactivity.

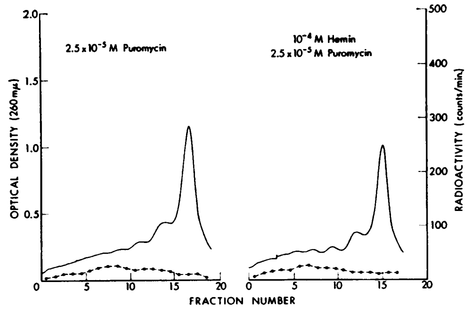

FIG. 4.—Incubation for 30 min with puromycin, and with puromycin plus hemin. ————, Optical density; – – – –, radioactivity.

cobalt to these cells results in an increase in the size and proportion of polyribosomes and in the radioactivity bound to them. These effects of cobalt are not prevented by desferrioxamine.

The effects of hemin on polyribosome aggregation were explored further in studies with various inhibitors of protein synthesis (Figs. 4, 5, and 6). The addition of hemin did not influence the partial disaggregation of polyribosomes and the marked inhibition of polypeptide chain formation which occur on incubation of the cells with puromycin (2.5×10^{-5} *M*) (Fig. 4). Nor did it alter the effects of cycloheximide (Fig. 5). Following the disruption of polyribosomes by incubation with sodium fluoride,[11] the addition of cycloheximide markedly inhibited polypeptide chain formation despite partial re-formation of polyribosomes; the addition of hemin did not enhance polyribosome formation or polypeptide synthesis. In the presence of o-fluorophenylalanine (2×10^{-3} *M*), however, the synthesis of soluble hemoglobin was inhibited by only 25 per cent, and the addition of hemin increased both polyribosome formation and globin synthesis (Fig. 6). The relevance of these observations to the mechanism of action of heme is discussed below.

To investigate the possible role which a shift in ribosomal subunits might play in the aggregation of polyribosomes by hemin, the proportion of ribosomal subunits in iron-deficient cells was examined by centrifuging sucrose gradients for 16 hr at 18,000 rpm. There were no significant differences in the proportion of subunits when lysates from iron-deficient reticulocytes were compared with lysates from reticulocytes obtained from acetylphenylhydrazine-treated rabbits. In iron-deficient cells the proportion of subunits was not altered when polyribosomal aggregation was stimulated by hemin nor when polyribosome breakdown was caused by desferrioxamine.

Discussion.—The effects of adding hemin to iron-deficient rabbit reticulocytes incubated with an isotopically labeled amino acid are: (1) a shift of single ribosomes to polyribosomes with an increase in the proportion and in the size of the polyribosomes, (2) an increase in the stability of the polyribosomes, (3) an increase in the specific activity of the polypeptide chains attached to the polyribosomes,[11a] and (4) an increase in the specific activity of the soluble hemoglobin. These results focus attention on the control of protein synthesis by mechanisms which regulate the size, stability, and functional activity of polyribosomes.

A functioning polyribosomal structure consists of ribosomes, messenger RNA, and sRNA-amino acid complexes, and it requires guanosine triphosphate and energy.

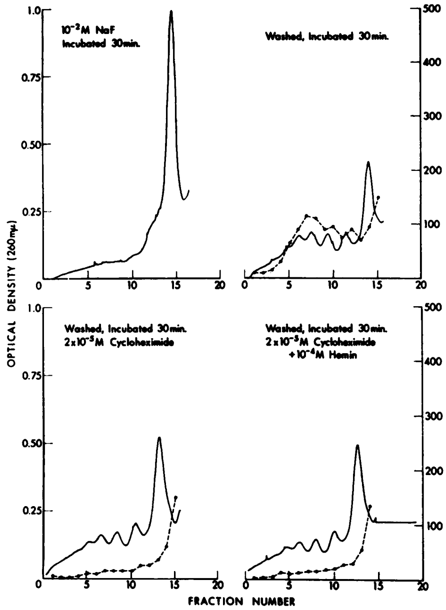

FIG. 5.—Incubation with NaF[11] for 30 min followed by washing three times with cold 0.15 M NaCl and reincubation for 30 min in standard medium containing 300 mg/100 ml glucose, with no further additions, with cycloheximide, and with cycloheximide plus hemin. ————, Optical density; – – – –, radioactivity.

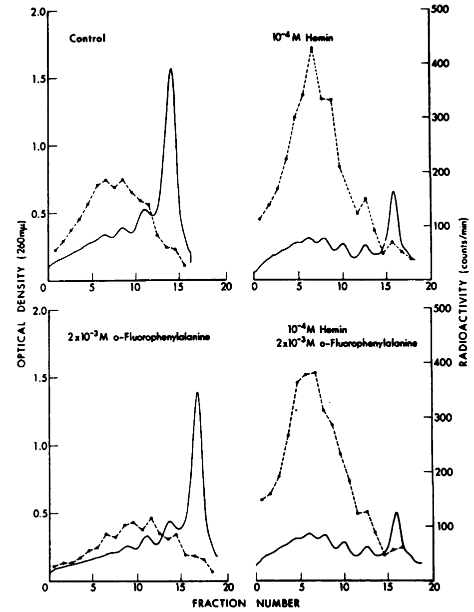

FIG. 6.—Incubation for 30 min with no additions (control), with hemin, with o-fluorophenylalanine, and with o-fluorophenylalanine plus hemin. ————, Optical density; – – – –, radioactivity.

Accordingly, there are several possible mechanisms of action of heme which may be considered.

Heme may attach to nascent chains of globin on the polyribosomes and may promote conformational changes in the polypeptide chains or in the polyribosome-messenger RNA-polypeptide chain complex. Such conformational changes might accelerate the movement of ribosomes along the messenger RNA, might thereby increase the availability of mRNA for attachment of single ribosomes, and could thus increase the proportion and size of polyribosomes. Conformational changes may also be invoked as a possible explanation of enhanced stability of the polyribosome complex. To test the validity of this hypothesis, we are trying to determine whether or not heme attaches to the growing polypeptide chain on the polyribosomes.

The suggestion that heme or protoporphyrin enhances the release from ribosomes of newly completed chains of globin has been made by Gribble and Schwartz[12] on the basis of their findings in a cell-free system. Another possible mechanism involves the salvage by heme of newly formed chains that might otherwise be destroyed. It is known that heme can promote the conversion of dimers of globin to the stable hemoglobin tetramer.[13] Enhanced release or salvage of newly completed chains could account for an increase in the specific activity of the soluble hemoglobin. But to explain the increase which we have observed in the specific activity of the chains on the polyribosomes, one would have to invoke an additional mechanism of feedback inhibition of protein synthesis by newly completed chains. As yet, evidence of such feedback control is not available. Neither enhanced release nor salvage would account for the observed increase in the proportion and size of polyribosomes.

Earlier studies[8, 9] have demonstrated enhanced hemoglobin formation in rabbit reticulocytes on the addition of iron, and more recent studies[1, 3] have shown the stimulatory effect of heme on the synthesis of globin. Our experiments with desferrioxamine indicate that heme exerts its stimulatory effect directly and not merely as a source of iron. It is likely that iron exerts a stimulatory effect by serving as a substrate for the synthesis of heme and by stimulating the synthesis of protoporphyrin.[9] The possibility remains, however, that iron may have another effect which is independent of its role in heme synthesis. Waxman and Rabinovitz[14] have reported the stabilization by iron of polyribosomes in reticulocytes. In our studies with cobalt, which inhibits the synthesis of heme,[10] effects on polyribosomes are observed which are very similar to those of iron. The role of heavy metals, such as iron and cobalt, in the structure of ribosomes and in the aggregation and stabilization of polyribosomes is now under study.

The lack of effect of hemin in cells treated with puromycin and cycloheximide, as compared to its effect in the presence of o-fluorophenylalanine, suggests that agents which intervene structurally in the polyribosome complex may not only inhibit protein synthesis but may also inhibit the action of heme. The disruptive effects of sodium fluoride emphasize the energy requirement of polyribosomes. The extent to which the effects of hemin, iron, and cobalt may be ascribed to effects on the energetics of the reticulocytes remains to be evaluated.

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[11a] To observe this increase, which reflects a faster rate of protein synthesis in the hemin-treated cells, it is necessary to label for periods as short as 2 min. With longer periods of labeling, e.g., 10 min or more, the specific activities of the nascent chains of control and of hemin-treated cells tend to be equalized, but the increased rate of synthesis continues to be demonstrable in the higher specific activity of the soluble hemoglobin of the hemin-treated cells.

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